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Isolation and characterization of a novel bat coronavirus closely related to the direct progenitor of SARS coronavirus

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Abstract

We report the isolation and characterization of a novel bat coronavirus which is much closer to the SARS coronavirus (SARS-CoV) in genomic sequence than others previously reported, particularly in the S gene. Cell entry and susceptibility studies indicated that this virus can use ACE2 as receptor and infect animal and human cell lines. Our results provide further evidence of bat origin of the SARS-CoV and highlight the likelihood of future bat coronavirus emergence in humans.

Key words: bat, SARS-like coronavirus, natural reservoir, receptor

Text

The 2002-3 outbreak of severe acute respiratory syndrome coronavirus (SARS-CoV) was a significant public health threat at the beginning of the twenty-first century (1). Initial evidences showed that the masked palm civet (*Paguma larvata*) was the primary suspect of the animal origin of SARS-CoV (2, 3). Later studies suggested that Chinese horseshoe bats are natural reservoirs and masked palm civet most likely served as an intermediate amplification host for SARS-CoV (4, 5). From our longitudinal surveillance of bat SARS-like coronavirus (SL-CoV) in a single bat colony of the species *Rhinolophus sinicus* in Kunming, Yunnan Province, China, we found a high prevalence of diverse SL-CoVs (6). Whole genome sequence comparison revealed these SL-CoVs have 78%-95% nucleotide (nt) sequence identities to SARS-CoV with the major difference located in the spike protein (S) genes and the ORF8 region. Significantly, we have recently isolated a bat SL-CoV (WIV1) and constructed an infectious clone of another strain (SH014) which are closely related to SARS-CoV and capable of using the same cellular receptor (angiotensin-converting enzyme, ACE2) as for SARS-CoV (6, 7). Despite the high similarity in genomic sequences and receptor usage of these two strains, there is still some difference at N-terminal domain of the S proteins between SARS-CoV and other SL-CoVs, indicating that more similar viruses are circulating in bat (s).

Here we report the isolation of a new SL-CoV strain, named bat SL-CoV WIV16. SL-CoV WIV16 was isolated from a single fecal sample of *Rhinolophus sinicus* which was collected in Kunming, Yunnan Province, in July, 2013. The full

genomic sequence of SL-CoV WIV16 (GenBank number: KT444582) was determined and contained 30,290 nt in size and a poly (A) tail, which is slightly larger than that of SARS-CoVs and other bat SL-CoVs (6, 8-13). The WIV16 genome has a 40.9% G+C content and short untranslated regions (UTRs) of 264 and 339 nt at the 5' and 3' termini, respectively. Its gene organization is identical to WIV1 and slightly different from the civet SARS-CoV and other bat SL-CoVs due to an additional ORF (name ORFx) detected between the ORF6 and ORF7 genes of the WIV1 and WIV16 genomes (data not shown). The conserved transcriptional regulatory sequence was identified upstream ORFx, indicating this is likely to be a potential functional gene. The overall nt sequence of WIV16 shared 96% identity, higher than any previously reported bat SL-CoVs, with human and civet SARS-CoVs (**Table 1**) (4-6, 8-13). A detailed comparison of protein sequences between the SARS-CoV GZ02, a strain from an early phase patient, and all reported bat SL-CoVs indicated that WIV16 is the closet progenitor of the SARS-CoV in most proteins, particularly in the S protein (**Table 1**).

The S protein is responsible for virus entry and is functionally divided into two domains, denoted S1 and S2. The S1 domain is involved in receptor binding and the S2 domain for cellular membrane fusion (14). S1 is functionally subdivided into two domains, an N-terminal domain (S1-NTD) and a C-domain (S1-CTD), both of which can bind to host receptors and hence function as receptor-binding domain (RBDs) (15). All isolates of SARS-CoV and SL-CoV share high identity in both nt and amino acid (aa) sequences in S2 region but highly diverse in their S1 regions. The WIV16 S

gene shared 95% sequence identity at nt level and 97% at aa level, respectively, with SARS-CoVs, much higher than that of WIV1 with 88% at nt level and 90% at aa level, respectively. Different from other bat SL-CoVs, the S1-NTD of WIV16 is much more similar to that of SARS-CoV (**Fig. 1**). The S1-NTD of WIV16 shared aa sequence identity of 94% with SARS-CoVs, but only 50%-75% with other bat SL-CoVs. It's worth to note that the WIV16 RBD (aa 318-510) shared 95% sequence identity with SARS-CoV, but is almost identical with WIV1. Thus WIV16 S gene is likely a recombinant of WIV1 and a recent ancestor of SARS-CoV.

High sequence conservation of the WIV16 RBD with that of SARS-CoVs predicts that WIV16 is likely to also use ACE2 as a cellular entry receptor. This was confirmed by infection of HeLa cells expressing ACE2 from human, civet and Chinese horseshoe bat, respectively (**Fig. 2A)**. Cell susceptibility test using different cell lines further indicated that WIV16 has the same host range as WIV1 (**Fig. 2B**) (6).

To assess whether the major sequence difference of the S1-NTD will have an effect on virus entry and/or replication, the growth kinetics of the two viruses was comparatively studied. Vero E6 cells were infected with WIV1 or WIV16 at MOI of 1 and virus production in the medium supernatant was determined at four time points post infection by quantification of viral RNA (**Fig. 3**, see figure legend for more technical detail). The two viruses grew at a very similar rate with WIV16 slightly slower than WIV1 during the 48-hr duration examined in this study. It is hard to conclude whether this subtle difference is significant and related to the S1-NTD

sequence difference. Further investigation with more cell lines is required to confirm this preliminary observation.

In conclusion, we isolated and characterized a novel bat SL-CoV isolate WIV16 which is the closest ancestor to date of the SARS-CoV. Our results provide further evidence that Chinese horseshoe bats are natural reservoirs of SARS-CoVs. It should be noted that the WIV16 is not the closest strain to the human SARS-CoVs with regards to ORF8. A full-length ORF8 is present in several SARS-CoV genomes of early phase patients, all civet SARS-CoVs and bat SL-CoVs. It is split into two ORFs (ORF8 a & b) in most of human SARS-CoVs from late phase patients due to a deletion event in this part of the genome (3). Recently two papers reported that they found a full-length ORF8 which share higher similarities to the SARS-CoV GZ02 and civet SARS-CoV SZ3, suggesting that SAS-CoV derived from a complicated recombination and genetic evolution among different bat SL-CoVs (10, 12). Taking together, we predict that there are diverse SL-CoVs to be discovered in bats. Continued surveillances of this group of viruses in bats will be necessary and important not only for better understanding of spill over mechanism, but also for more effective risk assessment and prevention of future SARS-like disease outbreaks.

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124

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Figure legends

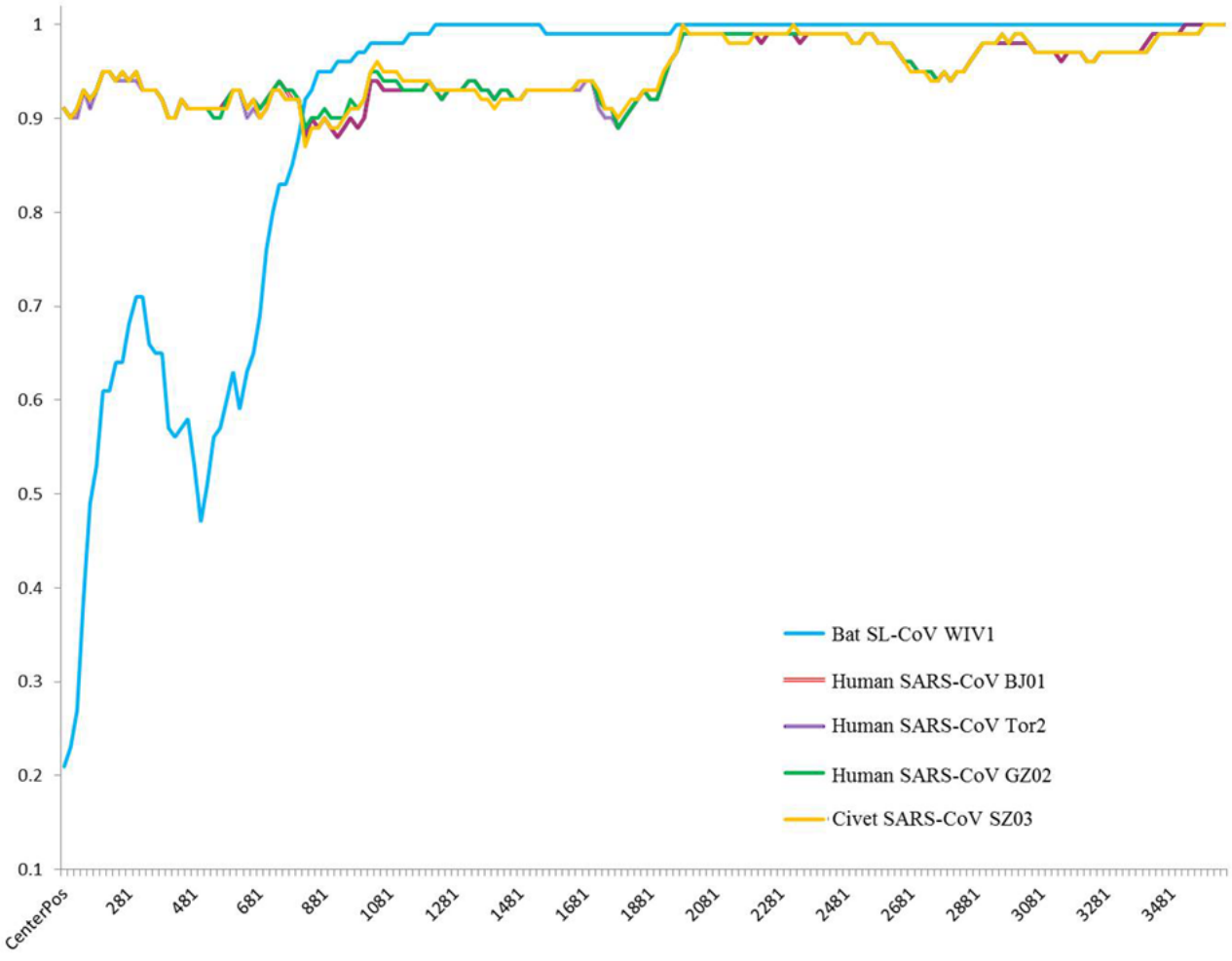
FIG 1 Similarity plot based on the nucleotide sequence of the S gene of bat SL-CoV WIV16. S genes of human/civet SARS-CoVs and bat SL-CoV WIV1 were used as reference sequences, with window of 200 bp, a step size of 20 bp, under Kimura model.

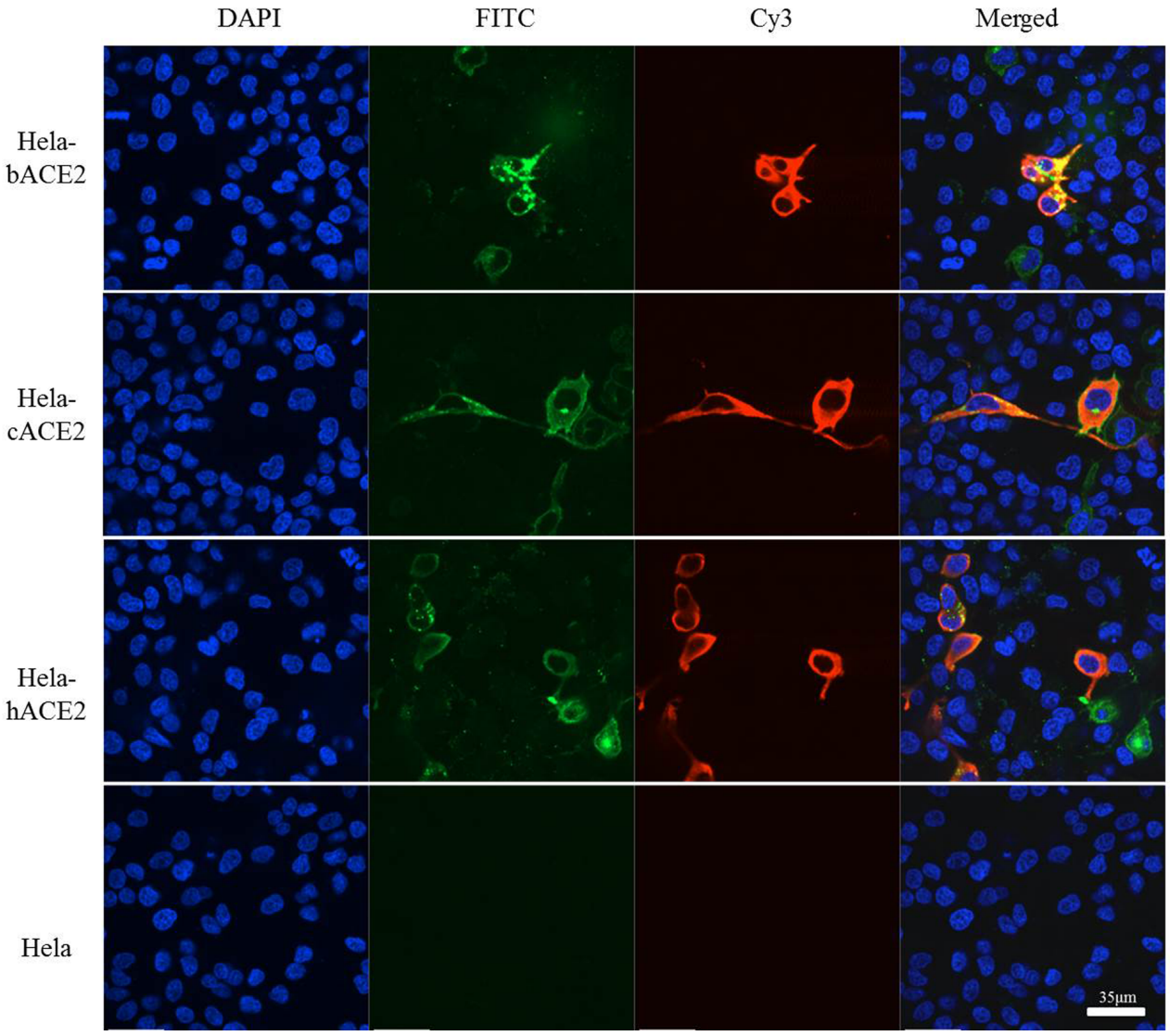
FIG 2 Receptor analysis (A) and susceptibility test (B) of bat SL-CoV WIV16.

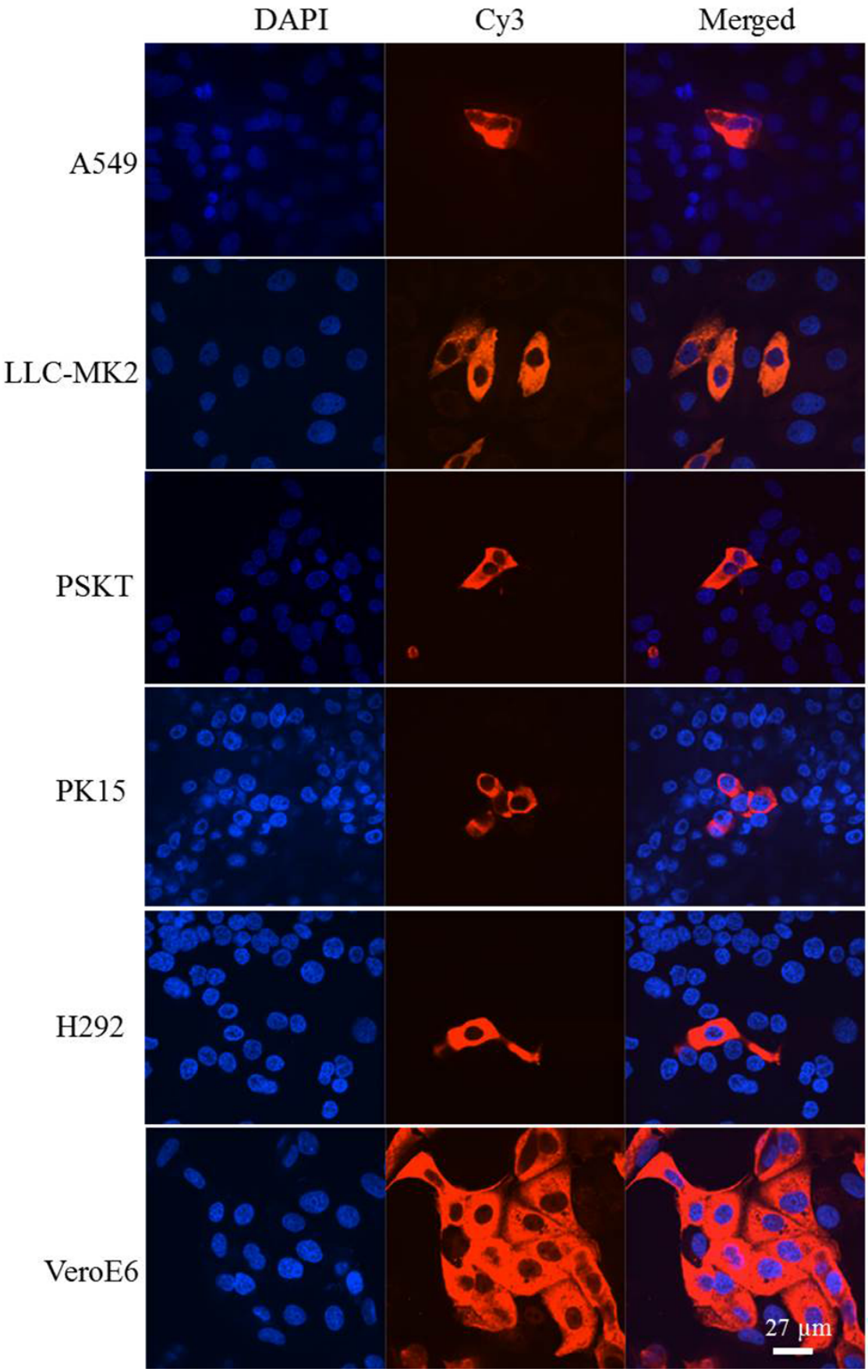
A, HeLa cells with and without the expression of ACE2. ACE2 expression was detected with goat anti-human ACE2 antibody followed by fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG. Virus replication was detected with rabbit antibody against the SL-CoV Rp3 nucleocapsid protein followed by cyanine 3 (Cy3)-conjugated mouse anti-rabbit IgG. Nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole). The columns (from left to right) show staining of nuclei (blue), ACE2 expression (green), virus replication (red) and the merged triple-stained images. b, bat; c, civet; h, human.

B, Virus infection in A549, LLC-MK2, RSKT, PK15, H292 and Vero-E6 cells. The columns (from left to right) show staining of nuclei (blue), virus replication (red), and the merged double-stained images. A549 and H292,human lung cells; LLC-MK2, macaque kidney cells; RSKT, Chinese horseshoe bat kidney cells; PK15, pig kidney cells; Vero-E6, African green monkey kidney cells

FIG 3 One-step growth curve of bat SL-CoV WIV16 compared with WIV1. Vero E6 cell was infected by WIV16 or WIV1 at an MOI of 1. Supernatants were collected at 0, 12, 24 and 48 h, post infection. The viruses in the supernatant were determined by one-step reverse real-time PCR (n=3), virus RNA that extracted from virus with known titer was used to set up the standard curve; error bars represent standard deviation.







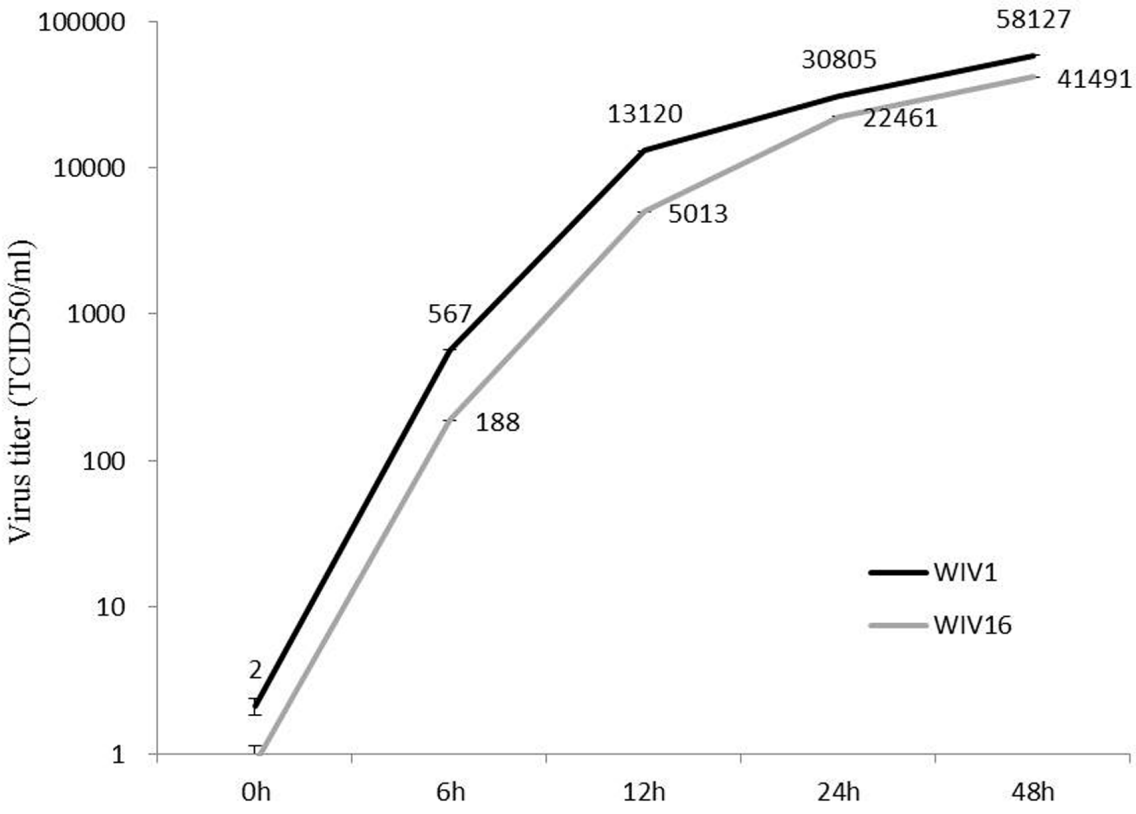


Table 1. Genomic comparison of SARS-CoV GZ02 with civet SARS-CoV and other bat SL-CoVs.

FL or ORFs	No. of nt	No. of aa	Identity nt/aa(%)											
			SZ3	WIV16	WIV1	Rs3367	RsSHC014	Rs672	Rp3	Rf1	Rm1	LYRa11	HKU3-1	YNLF_31C	BM48-31
FL	--	--	99.8	96.0	95.6	95.7	95.4	93.4	92.6	87.8	88.2	90.9	87.9	93.5	78.8
P1a	13,134	4,377	99.9/99.9	96.6/98.1	96.9/98.0	96.9/98.0	96.8/98.1	96.4/98.1	94.9/96.6	88.0/94.3	88.0/93.6	91.0/95.9	88.2/94.3	96.0/97.3	76.9/81.6
P1b	8088	2,695	99.9/99.9	96.1/99.1	96.3/99.4	96.3/99.4	96.4/99.5	96.0/99.3	96.2/99.1	90.9/98.3	91.4/98.6	93.8/98.9	90.9/98.6	96.8/99.2	85.5/96.0
S	3,768	1,255	99.6/99.0	95.4/97.3	90.2/92.4	90.2/92.5	88.4/90.2	77.6/80.1	78.1/80.2	75.5/78.4	78.0/80.6	83.3/89.9	77.0/79.4	76.1/79.2	70.9/76.0
(S1)*	2,040	680	99.5/98.8	92.6/95.4	83.3/86.5	83.4/86.8	79.9/82.4	68.8/67.0	69.1/66.7	66.7/66.1	69.0/67.4	80.3/84.4	69.2/67.2	67.5/66.7	65.8/64.5
(S2)*	1,728	575	99.8/99.3	98.3/99.5	98.3/99.5	98.2/99.3	98.3/99.5	88.0/95.5	88.4/96.2	85.5/92.7	88.3/96.0	87.3/96.3	85.9/93.9	86.0/93.7	76.7/89.6
ORF3a	825	274	99.0/97.8	99.2/98.2	99.0/97.8	99.2/98.2	99.3/98.2	90.4/90.8	84.0/84.3	88.6/86.9	83.5/84.3	89.7/91.6	83.0/82.5	89.0/88.3	73.1/71.5
E	231	76	100.0/100.0	99.1/100.0	99.1/100.0	99.1/100.0	98.7/98.7	99.6/100.0	97.8/100.0	96.5/96.1	96.1/98.7	98.3/98.7	97.4/100.0	99.6/100.0	90.0/92.1
M	666	221	99.8/99.5	97.4/98.2	97.4/98.2	97.4/98.2	97.4/97.7	97.7/98.6	93.4/97.3	95.5/97.7	94.7/97.3	94.7/97.7	95.0/98.6	95.9/98.6	81.5/91.4
ORF6	192	63	100.0/100.0	95.3/92.1	95.8/93.7	97.9/96.8	97.4/96.8	97.4/98.4	94.8/92.1	94.8/93.7	94.8/92.1	94.3/95.2	94.8/93.7	92.7/88.9	65.1/50.0
ORF7a	369	122	100.0/100.0	94.3/95.1	94.9/95.1	94.9/95.9	94.6/95.9	94.3/95.9	93.8/95.1	92.1/91.8	93.0/93.4	93.2/94.3	93.0/94.3	96.7/96.7	63.9/58.5
ORF7b	135	44	100.0/100.0	96.3/93.2	95.6/93.2	95.6/93.2	96.3/93.2	95.6/93.2	96.3/93.2	94.1/90.9	95.6/93.2	86.7/90.9	92.6/93.2	97.0/93.2	65.0/70.0
ORF8	369	122	99.5/98.4	50.1/38.6	50.7/39.5	50.7/39.5	50.7/40.4	51.6/39.5	53.3/39.5	82.1/81.8	52.1/39.5	51.0/38.3	52.1/37.7	82.1/82.6	N/A
N	1,269	422	99.9/100.0	98.4/99.5	98.4/99.8	98.7/100.0	98.3/99.5	97.6/98.6	96.7/98.1	94.2/95.7	96.4/97.9	96.9/97.9	96.2/96.7	97.2/98.3	78.5/88.2

SARS-CoV GZ02 was isolated from patients of early phase of the SARS outbreak in 2003. SARS-CoV SZ3 was identified from *Paguma larvata* in 2003 collected in Guangdong, China. SL-CoV WIV16, WIV1, Rs3367 and RsSHC014 were identified from *Rhinolophus sinicus* collected in Yunnan, China, during 2011 to 2013. SL-CoV YNLF_31C was identified from R. *ferrumequinum* collected in Yunnan, China, in 2013. SL-CoV LYRa11 was identified from R. *affinis* collected in Yunnan, China, in 2011. SL-CoV Rs672, Rp3 and HKU3-1 were identified from R. *sinicus* collected in China (respectively: Guangxi, 2004; Guizhou, 2006; Hong Kong, 2005). Rf1 and Rm1 were identified from R. *ferrumequinum* and R. *macrotis*, respectively, collected in Hubei, China, in 2003. Bat SARS-related CoV BM48-31 was identified from R. *blasii* collected in Bulgarian in 2008. FL, full-length genome. *S1, the N-terminal domain of the S protein (aa 1-680). S2, the C-terminal domain of the S protein (aa 681-1255). The pairwise comparison was conducted for all ORFs at nucleotide acids (nt) and amino acids (aa) levels. The full-length genome was compared at nt level. N/A, not available.